Periplasmic proteins encoded by VCA0261–0260 and VC2216 genes together with copA and cueR products are required for copper tolerance but not for virulence in Vibrio cholerae

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The bacterial pathogen Vibrio cholerae requires colonization of the human small intestine to cause cholera. The anaerobic and slightly acidic conditions predominating there enhance toxicity of low copper concentrations and create a selective environment for bacteria with evolved detoxifying mechanisms. We reported previously that the VCA0260, VCA0261 and VC2216 gene products were synthesized only in V. cholerae grown in microaerobiosis or anaerobiosis. Here we show that ORFs VCA0261 and VCA0260 are actually combined into a single gene encoding a 18.7 kDa protein. Bioinformatic analyses linked this protein and the VC2216 gene product to copper tolerance. Following the approach of predict-mutate and test, we describe for the first time, to our knowledge, the copper tolerance systems operating in V. cholerae. Copper susceptibility analyses of mutants in VCA0261–0260, VC2216 or in the putative copper-tolerance-related VC2215 (copA ATPase) and VC0974 (cueR), under aerobic and anaerobic growth, revealed that CopA represents the main tolerance system under both conditions. The VC2216-encoded periplasmic protein contributes to resistance only under anaerobiosis in a CopA-functional background. The locus tag VCA0261–0260 encodes a copper-inducible, CueR-dependent, periplasmic protein, which mediates tolerance in aerobic, but under anaerobiosis its role is only evident in CopA knock-out mutants. None of the genes involved in copper homeostasis were required for V. cholerae virulence or colonization in the mouse model. We conclude that copper tolerance in V. cholerae, which lacks orthologues of the periplasmic copper tolerance proteins CueO, CusCFBA and CueP, involves CopA and CueR proteins along with the periplasmic Cot (VCA0261–0260) and CopG (VC2216) V. cholerae homologues.

INTRODUCTION

The Gram-negative bacterium Vibrio cholerae is the aetiological agent of the severe human diarrhoeal disease cholera. Besides infecting humans, this pathogen survives epidemic and inter-epidemic cycles in riverine and estuarine waters (Faruque et al., 2004). The survival systems used by the vibrios inside and outside the human host are relevant scientific issues and their disclosure has been facilitated by the availability of the complete genome sequence of this bacterium (Heidelberg et al., 2000), together with improved methodologies for systems biology studies. We previously examined the effect of aerobiosis, anaerobiosis and microaerobiosis on the proteome of V. cholerae by 2D gel electrophoresis (combining IEF and SDS-PAGE) and MS analyses (Marrero et al., 2009). One relevant finding was that anaerobic growth promoted the synthesis of colonization factors encoded by the vibrio

Abbreviation: RT-PCR, reverse transcriptase PCR.
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pathogenicity island in \textit{V. cholerae} El Tor. Also interesting was the finding that the hypothetical proteins encoded by VCA0260, VCA0261 and VC2216 were only expressed under hypoxic conditions. Peptides corresponding to VCA0260 and VCA0261 proteins were identified from a single protein spot, suggesting that both ORFs coded for a single product. In addition, the predicted gene products of these two ORFs were similar to the N- and C-halves of a single putative copper-binding protein in \textit{Vibrio paraheamolyticus} (Marrero \textit{et al.}, 2009) and to the copper-tolerance protein Cot in \textit{Pseudomonas fluorescens} DF57 (Tom-Petersen \textit{et al.}, 2001), respectively. Since the \textit{cot} homologue PA2807 is the most upregulated gene in copper-adapted \textit{Pseudomonas aeruginosa} PA01 and its disruption leads to a slight reduction in copper tolerance (Teitzel \textit{et al.}, 2006), we decided to study copper homeostasis in \textit{V. cholerae}. Connected with this, the periplasmically located peptide encoded by VC2216 has been assigned putative cation resistance functions (von Krüger \textit{et al.}, 2006). The gene product is also similar to the Ralstonia \textit{metallidurans} CopG protein involved in copper resistance (Monchy \textit{et al.}, 2006). Thus, it is reasonable to hypothesize that the VC2216-, VCA0260- and VCA0261-encoded proteins are parts of copper-tolerance systems of \textit{V. cholerae} for which putative roles have not been experimentally investigated.

Copper is an essential trace element required as a cofactor for cellular enzymes (Rubino \& Franz, 2012). Nevertheless, micromolar copper concentrations are toxic to cells (Macomber \& Imlay, 2009), which is why intracellular copper levels are tightly controlled by homeostatic mechanisms (Osman \& Cavet, 2008). In the human digestive tract, copper concentration is not likely to exceed 10 $\mu$M (Rensing \& Grass, 2003); however, the acidic and anaerobic conditions prevailing there are known enhancers of copper toxicity (Weissman \textit{et al.}, 2000). Thus, intestinal pathogens have evolved systems to manage copper excess and its toxicity to survive these \textit{in vivo} conditions (Espariz \textit{et al.}, 2007; Rensing \& Grass, 2003; Weissman \textit{et al.}, 2000). In enteric Gram-negative bacteria, copper surplus is primarily handled by the cue regulon, which is controlled by the MerR family regulator CueR (Espariz \textit{et al.}, 2007; Outten \textit{et al.}, 2000). CueR activates the expression of copper homeostasis genes, such as those encoding PIB1-type ATPases, multicopper oxidases and metallochaperones. PIB1-type ATPases are the main copper tolerance system in the cytosol (Outten \textit{et al.}, 2000; Stoyanov \textit{et al.}, 2003). They are highly conserved and only the regulator CueR- and ATPase-encoding genes are present in all cueR-containing genomes (Pérez Audero \textit{et al.}, 2010). In contrast, a wider variety of periplasmic systems conferring copper resistance have been described, which have dissimilar roles under different oxygen concentrations. Under aerobic conditions, \textit{Escherichia coli} and \textit{Salmonella} use a multicopper oxidase (CueO and CueD, respectively) to oxidize periplasmic Cu(I) to the less toxic Cu(II) form (Lim \textit{et al.}, 2002; Singh \textit{et al.}, 2004). Under anaerobiosis, when the multicopper oxidases are inactive, \textit{E. coli} utilizes the CusRS-regulated CusCFBA transporter, to expel periplasmic Cu$^+$ ions (Outten \textit{et al.}, 2001). In contrast, \textit{Salmonella}, which contains no CusCFBA orthologues (Espariz \textit{et al.}, 2007), employs the CueR-dependent, copper-binding CueP protein (Osman \textit{et al.}, 2010; Pontel \& Soncini, 2009).

Being a human intestinal pathogen, \textit{V. cholerae} is expected to require tolerance systems to survive the copper stress in the gastrointestinal tract. To date, we have found no studies reporting these systems in \textit{V. cholerae}. In this study, we aimed to explore whether the overlapping VCA0261 and VCA0260 genes constitute a single gene and to determine the relevance of its gene product, as well as that of VC2216, in copper tolerance in \textit{V. cholerae}. Furthermore, we wanted to determine how they integrate into the remaining gene products of the network that might function in the bacterium. The ultimate aim of this study was to promote a basic understanding of the adaptive response mounted by this micro-organism against antimicrobial properties of copper in the human host, and during transmission and survival in the natural environment.

**METHODS**

**Bacterial strains and growth conditions.** \textit{E. coli} JM109 (Promega), BL21 (DE-3) (Novagen) and S17.1/pir (Simon \textit{et al.}, 1983) were used as cloning hosts. \textit{V. cholerae} O1 El Tor C7258 and N16961 strains were used for genetic manipulations. Bacterial strains were grown at 37 °C in Luria–Bertani (LB) broth or in syncaze medium (Marrero \textit{et al.}, 2009). When necessary, ampicillin and IPTG were added at 100 µg ml$^{-1}$ and 0.1 mM, respectively. Anaerobic conditions were generated in anaerobic jars using AnaeroGen sachets (Oxoid).

**Molecular biology techniques.** Standard cloning methods were used (Ausubel \textit{et al.}, 1995). Genomic DNA from C7258 was the template for PCR amplification, unless otherwise indicated. Automated DNA sequencing was subcontracted to Macrogen. Standard cloning methods were used for genetic manipulations. Bacterial strains were constructed by allelic replacement using derivatives of plasmid pCDV442 (Donnenberg \& Kaper, 1991), as previously described (Valle \textit{et al.}, 2000). Incorporation of mutations in constructed strains was confirmed by PCR and Southern blot hybridizations. For complementation studies, mutant strains were transformed by electroporation (Stoeberer \textit{et al.}, 1992) with plasmids carrying wild-type genes or empty control vectors.

**VCA0261 and VCA0260 gene cloning, expression, protein identification and specific antiseraum preparation.** Genes VCA0260 and VCA0261 were amplified by PCR in a single amplicon from \textit{V. cholerae} C7258 and N16961 using primers 5′-GCTCTAGAT-GGTTATTTTCAGGCC-3′ and 5′-AGTGTCAACAAATAGGAAG-AGG-3′, with XbaI and HinClI restriction sites, respectively. The amplicons were cloned as XbaI–HinClI fragments into pIJ2921 (Janssen & Bibb, 1993) to produce plasmids pIJN830-1 (C7258) and pIJN830-2 (N16961). Subsequently, VCA0261 and VCA0260 genes were subcloned as XbaI–BglII fragments into pET3a (Novagen), to generate the expression plasmids pET-N830-1 (C7258) and pET-N830-2 (N16961).

Protein expression was analysed by SDS-PAGE (Ausubel \textit{et al.}, 1995) in IPTG-induced, pET-N830-1 or pET-N830-2-containing BL21 (DE-3) cell lysates. Gels were stained with Coomasie blue (Ausubel \textit{et al.}, 1983) to visualize plasmids pCVD442 (Donnenberg & Kaper, 1991), as previously described (Valle \textit{et al.}, 2000). Incorporation of mutations in constructed strains was confirmed by PCR and Southern blot hybridizations. For complementation studies, mutant strains were transformed by electroporation (Stoeberer \textit{et al.}, 1992) with plasmids carrying wild-type genes or empty control vectors.
fingerprinting and the MS/MS ion search option of the MASCOT program, as previously described (Marrero et al., 2009). Additionally, gel strips containing the recombinant polypeptide were crushed and used to immunize New Zealand white rabbits, following standard procedures, to produce specific antisera. Animal studies were reviewed and approved by the Animal Experimental Ethics Committee of the National Centre for Scientific Research, Cuba.

Genetic constructs with mutated and wild-type alleles. Genes VCA0261 and VCA0260 were in-frame deleted by overlap expansion PCR, as described by Senanayake & Brian (1995). Briefly, VCA0261-upstream and VCA0260-downstream regions were independently amplified with primer pairs VCA0261-F (5'-GGATATCTGGGAAATATT-3')/VCA0261-R (5'-CTTACAGCTTTATCATAGTGTTAAGTTCTC-3') and VCA0260-F (5'-GATAAACGGCTAAGC GGAAATGTTGAAA-3')/VCA0260-R (5'-TGCTGCTCAAGTGCTGGGATATTGCTGGCCG-3'), respectively. The resulting products were pooled and reamplified with primers VCA0261-F and VCA0260-R. The final amplicon was blunt-cloned as an SpI–Hpal fragment into pIJ2921, obtaining plasmid pIJ-D.

The final amplicon was blunt-cloned as an HincII-excised kanamycin resistance cassette from pUC4K. Next, the deletion construct was cloned as a BglII–XbaI fragment into pCVD442 (a pCVD442 derivative containing a BglII site inserted into the Smal site), generating the deletion plasmid pCV–AVC0261–0260.

Since VC2215 overlaps VC2216 by 3 nt, an approximately 4.7 kb fragment containing both genes was amplified by PCR using the expand long template PCR system (Roche) and the primers 5'-CGGGATCCTAGGCTAAGATG-3' and 5'-GGATATCTGGGAAATATT-3'. The resulting amplicon was cloned into pGEM-4 plasmid vector (Promega), generating plasmid pGEM-VC2215–16. Then, gene VC2216 was inactivated at the Nhel restriction site by insertion of the HincII-excised kanamycin resistance cassette from pUC4K (Taylor & Rose, 1988), obtaining pGEM-VC2215–16-Kn. Next, the disrupted VC2216 gene was cloned as an XbaI–BamHI fragment into pCVD442 to produce plasmid pCV–VC2216-Kn.

Gene VC2215 in pGEM-VC2215–16 was in-frame deleted by removal of the BglII–XbaI fragment generating pGEM-AVC2215. Then, the mutated gene was cloned as a Nhel–SpI fragment into XbaI–Smal-digested pCVD442, creating plasmid pCV–AVC2215.

Moreover, a double VC2215–VC2216 mutant construct was obtained by deleting the BglII–Nhel fragment from pGEM-AVC2215–16. The deletion was marked at a BclI restriction site by ligation with the BglII-excised kanamycin resistance cassette from pUC4K. Next, the marked deletion was cloned into pCVD442 as an EcoRV–BamHI fragment, generating plasmid pCV–AVC2215–16-Kn.

Gene VC2216 was subcloned for complementation analyses. First, the VC2216 gene in pGEM–AVC2215 was cloned into pIJ2921 as an XbaI–SpI fragment. Then, the NcoI–BglII fragment containing VC2216 was cloned into pBAD/HisA, creating plasmid pBV2216.

Gene VC0974 was amplified by PCR using primers 5'-AGCATGACTGACGTTGCTGGGAAATATT-3' and 5'-GGGATATCTGGGAAATATT-3'. The EcoRI–PstI-digested PCR product was cloned into pIJ2922 (Janssen & Bibb, 1993), creating plasmid pVC0974. Subsequently, the VC0974 gene was mutated by deleting the 383 bp Clal–SacI fragment from pVC0974. The deletion construct was cloned as a BglII fragment into pCVD442, generating plasmid pCV–AVC0974.

Gene VCA0264 was amplified by PCR using the expand high fidelity plus PCR system (Roche) and primers 5'-AGTAGCTCACAATAGGAAAGAGG-3' and 5'-CTCTGTGCTAATGCGTAAAGG-3'. The HindII-digested PCR product was cloned into pIJ2921, giving plasmid pIJ-VCA0264. Subsequently, the VCA0264 gene was mutated by an in-frame deletion of the 648 bp XhoI–EcoRV fragment. Then, the mutated allele was cloned into pCVD442B as a BglII fragment, generating plasmid pCV–AVC0264.

Software for analysis of amino acid sequences. BLASTP and PSIBLAST software was used to search for homology to the peptide sequences. The databases EcoCyc (http://ecocyc.org/) and VchoCyc (http://bioyc.org/organism-summary?object=VCHO) were also used. The SignalP 4.0 (http://www.cbs.dtu.dk/services/) and the I-TASSER (Roy et al., 2010) servers were used for predicting the presence of signal peptides and the tertiary structure, respectively.

Copper susceptibility testing. Copper sensitivity was determined on synace medium, to be consistent with proteomic experiments carried out previously (Marrero et al., 2009). A 20 μl aliquot of a 10^-6 dilution from exponentially grown cells of the wild-type or each mutant strain was spread on synace plates containing increasing concentrations of copper. MIC values were determined after 24 or 48 h of incubation under aerobicosis or anaerobiosis, respectively.

Expression analyses of locus tag VCA0261–0260 and its protein in V. cholerae. Gene expression was analysed by reverse transcriptase PCR (RT-PCR). RNA was isolated using the high pure RNA isolation kit (Roche) from mid-exponential-phase cells of C7258 grown in synace medium, induced or not induced with 1 mM CuSO4 for 10 min. Reverse transcription was performed using the transcriptor first strand cDNA synthesis kit (Roche), following the manufacturer’s recommendations. Complementary DNA was amplified by PCR with primers 5'-TGGCTTTTCCACATTTCCGCG-3' and 5'-GTACCAACAGGATTGC-3' and Taq DNA polymerase (Roche). A dnaQ gene fragment was also amplified with primers 5'-GGCCACTATGTCCCAAGATC-3' and 5'-TCCCCGGTAAA CGIATAAC-3' as an internal control. Electrophoresed PCR products were analysed using the gene genius gel system (Syngen Synoptics). PCR products were normalized according to the amount of dnaQ detected in the same cDNA sample. Comparisons of relative intensities of PCR products, obtained from control and copper-exposed cells, were made using the two-sample t-test.

Protein expression was analysed by Western blotting. Mid-exponential-phase cells were induced with variable concentrations of several metal salts for different times. Proteins from whole-cell lysates were separated by SDS-PAGE (12%) and electrophoretically transferred onto a nitrocellulose membrane. For immunodetection, anti-VCA0261–0260 rabbit polyclonal antibodies and horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma) were used.

Cell fractionation. V. cholerae C7258 cells grown in the presence of 0.5 mM CuSO4 in LB broth for 1 h were fractionated in 0.1 M sodium phosphate (pH 7.0) and 0.25 M sucrose containing 2000 U polymixin B ml^-1. Spheroplasts were pelleted and resuspended in the original volume of SDS-PAGE loading buffer and the supernatant containing periplasmic components was diluted in 6 x SDS-PAGE loading buffer. Endogenous β-galactosidase was used as a cytoplasmic marker and was assayed with ONPG, as described by Zhang & Bremer (1995), in 1 ml whole cells or 100 μl treated cells. Colour density was read at 420 nm in a Shimadzu UV-160A spectrophotometer and Miller activity was calculated as 1000 × [A420/(OD600 of sampled culture x 1 ml x reaction time in min)].

Virulence and intestinal colonization assays in the infant mouse model of cholera infection. Virulence evaluation was done in 5-day-old Balb-C suckling mice by intragastric challenge with 50 μl aliquots from a suspension of ~10^8 exponentially grown V. cholerae cells ml^-1. Mice were returned to their mothers 4 h after dosing. Survival was followed daily over 5 days. At least 10 mice were used for each strain and each experiment was repeated twice. The survival of mice was plotted against time using Kaplan–Meier plots, and a log-rank test was performed to determine the statistical significance. For colonization assays, a dose of ~10^7 cells was delivered to infant mice...
Fig. 1. Sequence analyses of VCA0261–0260 and its products expressed in E. coli. (a) Genomic arrangement of genes VCA0264–VCA0254 of V. cholerae N16961 (Heidelberg et al., 2000). (b) Published and determined nucleotide sequences of the overlapping region VCA0261–VCA0260 for strains N16961 and C7258, respectively. Predicted amino acids are also shown. Underlined, start and stop codons of ORFs VCA0260 and VCA0261, respectively, for strain N16961. The guanine nucleotide, underlined in the VCA0261–VCA0260 sequence of strain C7258, was not reported in the N16961 published
sequence (indicated by the hyphen) and new amino acids are encoded as a result of nucleotide insertion (underlined sequence GSAV). (c) Deconvoluted ESI-MS spectra from tryptic digestion of induced bands in E. coli containing pET-N830-2 (N16961) or pET-N830-1 (C7258). Peaks labelled with asterisks or with a filled circle correspond to modified peptides containing methionine sulfoxide or pyroglutamic acid, respectively. The theoretical molecular mass corresponding to each signal is in parentheses, and the sequence assignment is indicated. (d) Deconvoluted ESI-MS/MS spectrum from the doubly charged peptide ([m/z 836.89] indicated with a solid arrow in (c), encoded by the junction region between VCA0261 and VCA0260.

and after a 24 h incubation, mice were euthanized and their intestinal contents were removed, homogenized and plated on LB agar for the enumeration of colonizing bacteria. The unpaired t-test was used to compare the total number of cells recovered from mice inoculated with the C7258 strain with those obtained from mice inoculated with mutant strains.

RESULTS

Genes VCA0260 and VCA0261 constitute a single gene encoding one protein in V. cholerae

Proteomic analysis of V. cholerae C7258 identified peptides corresponding to the VCA0260- and the VCA0261-encoded proteins from a single 21 kDa protein spot. Consequently, it was hypothesized that the overlapping genes VCA0260 and VCA0261 (Fig. 1a) constituted a single ORF in C7258 (Marrero et al., 2009). To test this, chromosomal regions spanning both genes from C7258 and N16961 were amplified by PCR, cloned into pET-3a vector (obtaining plasmids pET-N830-1 and pET-N830-2) and sequenced (GenBank accession no. AY972867). A comparison to the nucleotide sequence of the published genome of N16961 (Heidelberg et al., 2000) revealed one previously unidentified nucleotide located upstream of the stop codon of VCA0261 (Fig. 1b). This nucleotide shifts the reading frame of VCA0261 to the same frame of VCA0260, generating a larger ORF which encodes a protein with a predicted molecular mass of 18.7 kDa. Accordingly, protein profiles of IPTG-induced E. coli cells carrying plasmid pET-N830-1 or pET-N830-2 showed an intense band of approximately 20 kDa (data not shown). The ESI-MS spectra of tryptic peptides from induced bands in both recombinant clones were comparable (Fig. 1c), indicating similar amino acid sequences. The tryptic peptide [M + H]^+ = 1672.86 encoded by the overlapping region between VCA0261 and VCA0260 was identified (Fig. 1c) and sequenced by ESI-MS/MS analysis (Fig. 1d). The experimentally determined amino acid sequence completely matched the predicted peptide encoded by the DNA sequence (Fig. 1b, d), indicating that genes VCA0260 and VCA0261 are, in fact, a single unit (VCA0261–0260) in the examined V. cholerae strains. To further support this finding, BLASTp searches found a full-length and highly identical polypeptide in each recently sequenced genome of V. cholerae, but not in the old N16961 or 623-39 genomes (Table S1, available with the online version of this paper).

Proteins encoded by VCA0261–0260 and VC2216 genes, together with CopA and CueR, could mediate copper tolerance in V. cholerae

Preliminary BLASTp searches suggested that VCA0261–0260 and VC2216 products are the V. cholerae Cot (Vc-Cot) and CopG (Vc-CopG) homologues, respectively. Additional computational analyses revealed that the VCA0261–0260 protein belongs to the Cupredoxin family from the InterPro database (IPR008972). Cupredoxins adopt a Greek key β-barrel fold and use a type I copper site for electron transfer between partner proteins (Choi & Davidson, 2011). Accordingly, a prediction of the tertiary structure of the VCA0261–0260 protein with the I-TASSER server produced a model possessing an eight-stranded Greek key β-barrel fold and a putative single type I copper-binding site, formed by the conserved His98, Cys153, His158 and Met163 (Fig. S1a). On the other hand, the VC2216 protein belongs to the cluster of orthologues 3019 from the conserved domain database, which comprises predicted metal-binding proteins (Marchler-Bauer et al., 2011). This cluster was previously classified into the thioredoxin-like fold superfamily (Kinch et al., 2003). The structure calculation of the VC2216 product by the I-TASSER tool confirmed the reported structural predictions. In the structural models, the thiol-redox functional motif Cys-X-X-Cys is in close proximity to the other absolutely conserved residues Cys-His-Thr (Fig. S1b), in which His replaced the conserved thioltransferase Pro (Charbonnier et al., 1999). Mutation of the Pro residue to His in the VC2216 structurally related human thioredoxin leads to increased copper binding (Su et al., 2007). So, the conserved His77 in the VC2216 product may be a copper ligand as well. Thus, the computational structural analyses of VCA0261–0260 and VC2216 putative products also suggest that they may bind copper ions.

We performed additional BLAST analyses in the N16961 genome, looking for homologues of components of the E. coli copper regulons cue and cus. V. cholerae contains two orthologues of the cue regulon components: the transporter ATPase CopA (VC2215, http://www.uniprot.org/uniprot/Q9KPZ7) and the CueR regulator (VC0974, http://www.expasy.org/uniprot/P0C6D2). In line with their annotations, sequence analyses of their putative products showed that they present all canonical sequences of P (1B-1)-ATPase and CueR proteins (Tables S2 and S3). On the other hand, PSIBLAST analyses in the N16961 genome detected no orthologues of the periplasmic multicopper oxidase CueO.
from the cue regulon or components of the cus system. Since there is increasing evidence to indicate that the periplasm is an important cellular compartment for copper homeostasis (Bagai et al., 2008; Eglér et al., 2005; Hiniker et al., 2005; Macomber et al., 2007), elucidating the involvement of Vc-Cot (VCA0261–0260) and Vc-CopG (VC2216) homologues in V. cholerae copper tolerance represented an interesting topic of study. The evaluation of the role of VC2215 and VC0974 genes in copper resistance was also interesting, since their original annotations were only based on sequence similarity.

VCA0261–0260 and VC2216 genes, along with copA and cueR, are required for full copper tolerance of V. cholerae under aerobic and anaerobic growth

V. cholerae C7258 was genetically modified by mutating the putative copper tolerance genes and the mutants were examined for copper susceptibility as indicated above. Under aerobic growth, copper tolerance decreased below wild-type levels for mutants in the putative Vc-cot, Vc-cueR or Vc-copA homologues. The mutant in the putative copA homologue attained the stronger susceptibility (Table 1, Fig. 2a). Copper tolerance decreased further in the triple mutant of the putative copA, copG and cot homologues. These results support the idea that proteins encoded by the cot, cueR and copA genes play a role in copper tolerance with CopA having the major individual contribution. Copper susceptibility of single mutants in the Vc-cot and Vc-copA under aerobiosis was restored to wild-type levels by trans-complementation with the corresponding wild-type gene (Fig. 2c), indicating that the observed decrease in tolerance resulted from mutations in the specific target genes. Copper tolerance of the Vc-copG mutant remained at wild-type levels under aerobic growth (Table 1, Fig. 2a). In addition, double mutants in the putative copA/copG or cot/copG homologues were as copper sensitive as single mutants in the putative copA or cot homologues (Table 1).

These results suggest that in oxygenated environments, the Vc-copG homologue plays no role in copper resistance or that an alternative system may act under this condition. V. cholerae was more susceptible to copper in anaerobiosis than in aerobiosis (Table 1 and Fig. 2b), which concurs with previous reports for other bacteria (Espariz et al., 2007; Macomber & Imlay, 2009; Outten et al., 2001).

Fig. 2. Copper tolerance of V. cholerae strains under different conditions. Strains C7258 and mutants in genes VCA0261–0260 (cot), VC2216 (copG), VC2215 (copA), VC2215–VC2216 (copA/copG) or VC2215, VC2216 and VCA0261–0260 (copA/copG/cot) were grown on syncase medium (–Cu²⁺) or syncase medium supplemented with CuSO₄ under aerobiosis (a) or anaerobiosis (b). (c) Mutants in genes VCA0261–0260 (cot), VC2216 (copG) or VC2215 (copA) carrying plasmidic vectors or their derivatives with a wild-type copy of the corresponding mutated gene were cultured on syncase-ampicillin (–Cu²⁺) or syncase-ampicillin supplemented with CuSO₄ under aerobiosis (VCA0261–0260 and VC2215) or anaerobiosis (VC2216). In this last case, syncase medium was supplemented with 0.2% glucose and 0.2% arabinose. pIJ, pIJ2921; pBAD, pBAD-HisA; pGEM, pGEM-T and pG2215, pGEM-VC2215-16-Kn.
Transcription of Vc-cot is induced by copper and Vc-Cot synthesis is copper and CueR dependent

The transcriptional response of Vc-cot to copper was determined by RT-PCR. The mean relative intensities of PCR products from the corresponding mRNA (Fig. 3a) isolated from copper-challenged V. cholerae cells showed statistically significant differences (P=0.0153) compared with those from control cells. The relative intensity of the PCR product for Vc-cot-specific mRNA in copper-exposed cells was about fivefold higher than in control cells (Fig. 3b). These results suggest that Vc-cot gene expression is activated by copper. Accordingly, analysis of the Cot protein content of V. cholerae C7258 cells by immunoblotting showed an immune-reactive band with an apparent size of 20 kDa in copper-grown cells under aerobic and anaerobic conditions. The intensity of the reaction exhibited a dose-dependent response (Fig. 3c) and time course analysis under aerobic growth revealed a rapid and sustained production of Cot as the copper concentration increased (Fig. 3d). However, the specific polypeptide was not detected in ZnSO4- or NiSO4-grown C7258 cells (Fig. 3e). These results suggest that the protein is specifically expressed in response to copper. The Cot protein was also produced as a single 20 kDa band in copper-grown V. cholerae N16961, O395 and MO45 cells (Fig. S2), confirming the correctness of nucleotide sequencing in strain N16961 deposited with our culture collection and the VCA0261–0260 BLASTP results with the published complete genomes of O395 and MO45 isolates (Figs I and S1).

To investigate which regulator is involved in copper-activation of Vc-cot expression, protein synthesis was evaluated in copper-grown cueR (VCA0974) and VCA0264 (a cueR parologue located close to Vc-cot) mutant cells. Cot synthesis was abolished only in cueR cells (Fig. 3e), which recovered Cot synthesis after trans-complementation with a cueR wild-type copy (Fig. 3e). These results indicate that CueR may regulate, directly or indirectly, Vc-cot expression. Consequently, the 109 bp intergenic upstream region of Vc-cot (VCA0261–0260) was examined for the presence of a CueR-binding motif, using the consensus sequence of the CueR operator (Pontel & Soncini, 2009). A consensus sequence resembling the CueR box was not detected in this region but was detected 29 nt upstream of the VCA0262 predicted start codon (Fig. 3f) (Pérez Audero et al., 2010). Therefore, CueR may directly regulate Vc-cot expression by binding to this site.

Bioinformatics and SignalP analyses predicted that the Vc-cot product is periplasmically located (Table S4) and has a signal peptide of 20 residues, respectively. Therefore, its localization was studied in polymixin B-fractionated V. cholerae cells. A 20 kDa protein appeared almost exclusively in the polymixin B extracts from copper-grown cells (Fig. 4a), which was recognized as Vc-Cot by immunoblotting (Fig. 4b). This protein was not detected in the spheroplasts or periplasmic fractions of cells grown without copper. The specific activity of the cytoplasmic marker β-galactosidase in the spheroplast fraction was similar to that of the whole-cell extract and undetected in the periplasmic fraction (Fig. 4a).

CopA is required for V. cholerae copper tolerance at low copper concentrations under acidic conditions and not for virulence or colonization in the infant mouse intestine

The possible role of copA, copG and cot in the survival of V. cholerae in the presence of copper under acid conditions, which represent the gastric environment, was investigated.
Copper toxicity increased significantly in acid culture medium under aerobic conditions and it did not augment further under hypoxic conditions (data not shown). At pH 4.5, wild-type cells failed to survive above 10 μM CuSO₄; meanwhile, the copA mutant strain and its derivatives did not survive at 4 μM CuSO₄ (Fig. 5a). These results indicate that copA plays an essential role in copper tolerance under these conditions.

The in vivo requirement of copA, copG and cot genes for V. cholerae pathogenesis was analysed by studying virulence and intestinal colonization of the copA/copG double mutant and the copA/copG/cot triple mutant in the suckling mouse model of cholera infection. Log-rank analysis did not indicate a significant difference (P=0.7778) in the mean survival time of mice challenged with wild-type C7258 or each mutant strain. In addition, the differences in the mean c.f.u. recovered from infant mice intestines between each mutant and the wild-type strain did not achieve statistical significance: copA/copG versus wild-type (P=0.8855) and copA/copG/cot versus wild-type (P=0.2738) (Fig. 5b). These results were supported by competition experiments with the mutant and wild-type strains, showing similar content in mouse intestine for both the wild-type and mutant strains (data not shown). These results suggest that copper tolerance determinants cot, copA and copG are dispensable for V. cholerae pathogenesis during infection within the infant mouse intestine.

**DISCUSSION**

In the first annotation of the N16961 genome, only two genes were related to copper homeostasis (cutC–VC0730 and cutF–VC1962). In E. coli, cutC deletion (Rensing & Grass, 2003) leads to increased copper sensitivity at high
copper concentrations and recent studies have suggested that CucC may be a cytoplasmic copper-binding shuttle protein that plays a role in intracellular copper trafficking (Li et al., 2005). On the other hand, cutF encodes an outer membrane lipoprotein in E. coli (Gupta et al., 1995), which has an indirect connection with copper homeostasis by mediating copper activation of the CpxAR system (Hirano et al., 2006). In this study, we have shown that the E. coli copA and cueR homologues of V. cholerae (VC2215 and VC0974, respectively) are indeed involved in copper tolerance under aerobic and anaerobic growth and, accordingly, we propose to name them copA and cueR. CopA seems to be the main system used under both conditions (Table 1) and it probably protects the cytoplasm from copper toxicity by driving cytoplasmic Cu⁺ ion efflux into the periplasm, like in E. coli (Rensing et al., 2000). A putative CueR-binding site was identified upstream of the copA gene (Pérez Audero et al., 2010); therefore, its expression may be CueR-activated in the presence of copper, like in E. coli (Outten et al., 2000; Stoyanov et al., 2001). However, copper tolerance of the cueR (VC0974) mutant under aerobic and anaerobic conditions was higher than that of the copA (VC2215) mutant (Table 1), suggesting that copA has a high basal expression or it is controlled by another regulator. The genome of V. cholerae encodes another putative Cu⁺-ATPase (VC1437), which was classified as a FixL/CopA2-like Cu⁺-ATPase (González-Guerrero et al., 2010), but mutations of this type of ATPases do not lead to copper sensitivity in other organisms (Raimunda et al., 2011).

Furthermore, we have shown that the products of genes VC2216 and VCA0261–0260, which is composed of the overlapping genes VCA0261 and VCA0260, are differentially required for full copper tolerance of V. cholerae under aerobic and anaerobic growth. The VC2216-encoded and VCA0261–0260-encoded proteins are similar to CopG of R. metallidurans and to Cot of P. fluorescens, respectively; therefore, we propose to name them CopG and Cot, respectively. The Cot protein is a periplasmic protein (Fig. 4), just like the CopG polypeptide (von Krüger et al., 2006); therefore, they could be part of alternative periplasmic copper tolerance systems in V. cholerae, in which orthologues to CueO and CubCFBA from E. coli and CueP from Salmonella (Pontel & Soncini, 2009) were not identified.

At present, we do not know what the molecular functions of CopG and Cot proteins at the periplasm are, but they may bind copper ions, according to the computational structural analyses of their amino acidic sequences. We do not know whether they interact with other proteins to mediate copper tolerance. However, production of Vc-Cot depends on an active CueR (Fig. 3e) and Vc-cot expression is probably directed from a putative CucR-regulated promoter (Fig. 3f), found upstream of the VCA0262 gene (Pérez Audero et al., 2010). This gene encodes a putative

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**Fig. 4.** Cellular location of Cot in V. cholerae. (a) Coomassie blue-stained gel of polyvinyl B subcellular fractions from C7258 cells induced with 0.4 % lactose (−) or 0.4 % lactose plus 1 M CuSO₄ (+). (b) Western blot of the same samples with an antiserum against Vc-Cot protein. Fractions: P, periplasm; S, spheroplast. β-Galactosidase activity in periplasmic fractions or its percentage in spheroplast fractions with respect to that of whole-cell fractions under each condition (in non-induced cells, 22.02 ± 2.29 Miller units; in copper-induced cells, 10.58 ± 0.43 Miller units) are also shown. ND, Not detected.

**Fig. 5.** Role of V. cholerae copper tolerance determinants under different conditions. (a) Copper survival profiles of the wild-type C7258 strain and the copA, copA/copG and copA/copG/cot mutants under acidic anaerobic conditions. Approximately 1×10⁶ exponentially grown cells were used to inoculate replicated wells of a 96-well plate, containing 200 µl syncaze, pH 4.5, supplemented with salts and CuSO₄, which was then incubated at 37 °C for 20 h under anaerobiosis without shaking. (b) The colonization level of ~10⁹ c.f.u. wild-type C7258, copA/copG and copA/copG/cot mutant strains in the infant mouse model. C7258 (●), copA ( ○), copA/copG (□) and copA/copG/cot (△). Error bars represent SD.
periplasmic hypothetical protein according to a prediction from the SignalP 4.0 Server but its role in copper homeostasis is unknown. On the other hand, mutation of copG in a copA background led to no further decrease in copper tolerance under anaerobiosis (Table 1). This result suggests a physiological interaction between the periplasmic CopG protein (von Krüger et al., 2006) and CopA. This connection is also suggested at the genomic level, since orthologues of VC2216 are always found downstream of the ATPase-encoding gene copF (Monchy et al., 2006). In fact, one of the least studied aspects of the transport mechanism of Cu$^+$-ATPases is how the ion is released from the intramembrane metal-binding site into the periplasm (Barry et al., 2011; Raimunda et al., 2011). However, copper might not be found free in the periplasm and periplasmic copper chaperones have been described by Kim et al. (2010) and Robinson & Winge (2010). Thus, according to our results, the function of CopG in the periplasm may be closely related to that of CopA.

Although V. cholerae mutants in copper tolerance determinants were susceptible in vitro to physiologically relevant copper concentrations (10 μM), they were not attenuated for virulence in the infant mouse model, suggesting that these genes are dispensable for V. cholerae pathogenesis in this model. Consequently, copper tolerance determinants could be important in other settings. Thus, copA (VC2215) was implicated in biofilm formation in an environmental non-toxigenic V. cholerae strain (Mueller et al., 2007). On the other hand, it was recently hypothesized that the copper detoxification machinery has been conserved during evolution to provide protection against phagocytosis, either by host immune responses or predatory amoebae (Raimunda et al., 2011). V. cholerae O1 and O139 are able to multiply and survive inside the aquatic free-living amoeba Acanthamoeba castellanii (Abd et al., 2007) and Acanthamoeba polyphaga (Sandström et al., 2010). Therefore, it would be interesting to test whether copper tolerance determinants in V. cholerae play any role in survival within protozoas or during biofilm development.

In light of our results, we propose that copper tolerance in V. cholerae involves the coordinated action of several factors (Fig. 6). CueR (VC0974) senses copper and regulates the expression of the Cu(I)-translocating P-type ATPase, CopA (VC2215) and the Vc-Cot homologue (VCA0261–0260). While CopA pumps out excess copper ions from the cytoplasm, the periplasmic Cot protein is strongly overexpressed and would initially detoxify the copper burden rapidly in the periplasm by an undetermined mechanism, thereby preventing copper-mediated toxicity. However, under anaerobic conditions, although Cot is highly expressed, it may not be so active (as in aerobiosis), but metal binding by the V. cholerae periplasmic CopG homologue (VC2216), expressed from the VC2215 promoter, could detoxify periplasmic copper. At present, we do not know whether Cot and CopG proteins interact with other proteins to transfer copper ions outside the cell. Simultaneous mutation of periplasmic copper homeostatic determinants has a higher impact on copper tolerance in E. coli and Salmonella (Grass & Rensing, 2001; Pontel & Soncini, 2009). However, the cot/copG double mutant was as copper susceptible as the single cot and copG mutants under aerobiosis and anaerobiosis, respectively (Table 1). Therefore, V. cholerae must contain additional systems to handle periplasmic copper stress.

**Fig. 6.** Copper homeostasis in V. cholerae involves the coordinated action of CopA, CueR and the periplasmic proteins Cot and CopG. Question marks indicate that the mechanism used to transport copper inside the cell is unknown, as is that used by Cot and CopG to detoxify copper.

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